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Regenerate Limb Bud Sufficient for Claw Reversal in Adult Snapping Shrimps

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Abstract. The paired, bilaterally asymmetric snapper and pincer claws in the adult snapping shrimp *Alpheus heterochelis* were simultaneously autotomized at the beginning of an intermolt, and the resulting growth of the limb buds was characterized into several stages. At the next molt the limb buds emerged as newly regenerated claws of the same morphotype as their predecessors. Next, the paired claws were autotomized sequentially, with the second autotomy timed to different stages of limb bud growth at the first autotomy site. When the snapper is autotomized and a limb bud varying from stages 1 to 5 is allowed to develop at this site before the pincer is removed, the paired claws regenerate in their previous configuration. Similarly, claw asymmetry is retained when the pincer claw is removed first and an early limb bud (stage 1–2) is allowed to form at this site before the snapper is autotomized. However, claw asymmetry is reversed if an advanced limb bud (stage 3–5) is allowed to form at the pincer site before the snapper claw is removed. Under these conditions a snapper regenerates at the pincer site and a pincer at the snapper site. Because the limb bud at this pincer site regenerates as a snapper rather than a pincer, claw transformation has occurred, with the stage 3–5 limb bud substituting for an intact pincer. Therefore, the minimal requirement for pincer-to-snapper transformation is a stage 3–5 limb bud. We postulate that the newly transforming snapper claw restricts regeneration at the contralateral old snapper site to a pincer, thereby ensuring that claw bilateral asymmetry is present, albeit reversed.

Introduction

The first pair of thoracic chelipeds, or claws, in adult snapping shrimps of the Alpheid family are much larger than the remaining thoracic limbs and are bilaterally asymmetric, consisting of a pincer and snapper claw. The snapper is a much hypertrophied structure almost half the size of the entire animal and is specialized into a powerful snapping tool; a hammer on the dactyl plunges into a matching socket on the pollex, resulting in a loud popping sound (hence the name snapping shrimps) and a jet stream of water (Hazlett and Winn, 1966). The snapping behavior is used in agonistic encounters and also in crushing the shells of bivalves (McLaughlin, 1982). The contralateral pincer claw is smaller and used primarily in burrowing and feeding (Read and Govind, 1991).

Claw laterality, or handedness, is random in snapping shrimps, and the snapper appears with equal probability on the right or left side of the animal. However, handedness may be switched in adult shrimps. This happens when the snapper claw is removed at the beginning of an intermolt and in its place a new limb bud regenerates which at the next molt unfolds into a pincer claw, while the contralateral intact pincer claw is transformed into a snapper claw (Przibram, 1901; Wilson, 1903). When only the pincer claw is removed, a new pincer regenerates in its place; when both claws are removed, the regenerates appear in the same morphotype as their predecessors.

The latter procedure of removing both claws within an intermolt was used in an original and imaginative manner by Darby (1934), who varied the time interval between the two autotomies in the tropical shrimp *Alpheus armillatus*. These shrimps live off the coast of Bahama in ocean temperatures of 28–30°C and have an intermolt period of 10.5 days, or 252 h. His findings may be summarized as follows. In the experiment in which the snapper claw is removed first and then the pincer claw, despite varying the time interval between the two autotomies from 20–120 h, the paired claws regenerated in their previous configuration. Autotomy of the pincer claw beyond 120 h

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after autotomy of the snapper did not allow for a sufficiently advanced limb bud to form on the pincer site, and a claw failed to regenerate at this site at the next molt. Thus, sequential removal of first the snapper and then the pincer within a single intermolt was similar to simultaneous removal of both claws, as both procedures resulted in the regeneration of paired claws in their previous configuration (Wilson, 1903; Govind *et al.*, 1986).

In another series of experiments Darby (1934) removed first the pincer claw, soon after a molt, and then at varying time intervals the snapper. If the snapper claw was removed up to 29 h after pincer autotomy, the paired claws regenerated in their original configuration. Later removal of the snapper claw led increasingly to a reversal of claw asymmetry; *i.e.*, a pincer regenerated in place of the pristine snapper claw and a snapper regenerated in place of the pristine pincer claw. Thus, when the snapper claw was removed 33 h after pincer autotomy, reversal of asymmetry was seen in 50% of the animals; removal 40 h and 72 h after pincer autotomy produced reversal in 67% and 100% of the animals respectively. In these experiments in which the paired claws are autotomized sequentially, those with the shorter time interval between pincer and snapper autotomy are equivalent to removing both claws at the same time, because claw asymmetry is retained in the previous configuration, whereas those with the longer time intervals are equivalent to removing the snapper alone, because asymmetry is reversed. During these longer time intervals, what transpires that leads to reversal?

One of the events that transpires after a claw has been autotomized is the formation, at this site, of a limb bud that develops during the intermolt and emerges as a new claw at the next molt. Because reversal of claw asymmetry involves transformation of an existing pincer claw into a snapper, we considered the possibility that a limb bud may serve in place of an intact pincer as a suitable target for transformation. This would explain Darby's (1934) findings that claw asymmetry reverses at the longer time intervals between pincer and snapper autotomy, because a limb bud has had time to form at the pincer site. To test this hypothesis, we monitored the development of limb buds at the autotomy sites when both claws are removed simultaneously, producing a chart for limb bud growth. Using this growth chart, we repeated Darby's experiments but, rather than removing the second claw based on the time elapsed after removing the first claw, we removed the second claw at different stages of limb bud growth at the first autotomy site. We find that the presence of a sufficiently advanced limb bud at the pincer site when the snapper is autotomized leads to reversal of claw asymmetry, thereby explaining Darby's results. Additionally, our results demonstrate, for the first time, that the minimum requirement for pincer-to-snapper transformation is a limb bud.

Materials and Methods

Adult snapping shrimps of the species *Alpheus heterochelis* (Say) were collected from the tidal pools around Beaufort, North Carolina, and shipped to our laboratory in Scarborough, Ontario. The animals were held in 25-l glass aquaria equipped with a bottom gravel filter and partitioned into 12 compartments with fiberglass screens (Govind *et al.*, 1986). The aquaria were filled with artificial seawater that was kept at room temperature (22°C). A specially prepared diet—a blended mash of chicken livers and hearts, carrots and commercial cereal—was fed to the animals daily, and occasionally live food was provided in the form of *Tubifex* worms. The shrimps were sexed on arrival in the laboratory and their molt history during captivity was recorded.

Shrimps of both sexes were used, and only those with well-differentiated pincer and snapper claws were selected. These animals were allowed to molt twice before being used; this ensured that the claws were pristine, because earlier studies (Read and Govind, 1991) have shown that at least three intermolts are required for a newly regenerated limb to be fully differentiated. These claws are regarded as pristine in the present report (Fig. 1A). In our laboratory, the intermolt period was between 14 and 26 days (an average of 18.2 days) at 22°C.

Removal of a claw was accomplished by gently pinching the limb near the base of the merus, thereby inducing the animal to autotomize the claw at a preformed fracture plane. The following experiments were performed using this procedure.

(1) A day or two after a molt, the paired claws were removed within minutes of each other; this is regarded as

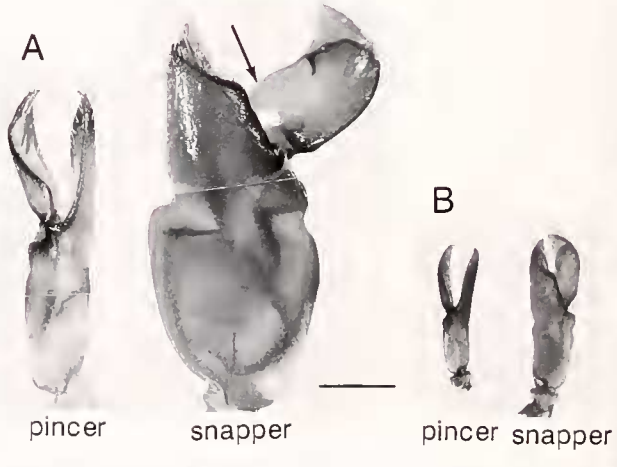

Figure 1. Representative pristine (A) and newly regenerated (B) pincer and snapper claws showing the hypertrophied snapper with the characteristic plunger on its dactyl (arrow). Pristine claws are asymmetric in size, whereas the newly regenerated ones are similar in size and substantially smaller. Scale 3 mm; magnification ×5.5.

simultaneous autotomy. The regenerating limb buds at both sites were monitored daily and sketches were made that were subsequently categorized into a series of developmental stages. The regenerating limb buds were also measured, in blind observations, under a dissecting microscope with a calibrated eyepiece. To reduce the chance of damaging the very delicate limb buds by repeated handling, measurements were made at 3–4 day intervals. Even so, 11 of the 27 animals suffered damage to the buds and were excluded from the study. To compensate for differences in animal size, limb bud measurements were expressed in terms of a regeneration (R) value where R = (limb bud length/carapace length) × 100 (Bliss, 1960).

(2) Within one or two days following a molt, the snapper claw was removed and the regenerating limb bud at this site was monitored daily. Next, the pincer claw was removed at varying stages of limb bud growth at the old snapper site. Of the 52 animals subjected to these sequential autotomies, 38 successfully regenerated paired claws at the next molt.

(3) The experimental protocol was similar to that in experiment 2, except that the pincer claw was removed first and at various stages of its limb bud growth the snapper claw was removed. Twenty-four of 36 animals successfully regenerated paired claws at the next molt.

Results

Stages of limb bud regeneration

Following autotomy of a claw, a new limb gradually regenerates at the base. The limb bud that forms is covered by a tough flexible cuticular coat that persists throughout the intermolt and is discarded only at the molt. Limb bud formation begins immediately as a small papilla (stage 1) that enlarges into an apical blastema (stage 2) (Fig. 2). The blastema elongates and acquires a club-like appearance distally. In the next stage (stage 3), a longitudinal furrow appears in the distal tip, marking the beginning of segmentation by dividing this region into the putative dactyl and propus segment. This is followed by the appearance of a series of transverse furrows along the length of the limb bud (stage 4). By stage 5, segmentation is complete and the limb has acquired typical pincer-like proportions. Further differentiation into a snapper-type claw is marked by the characteristic appearance of a plunger on the dactyl and a matching socket on the pollex (stage 6) and, in the case of a pincer-type claw, the appearance of a fringe of hair on the propus and dactyl of male shrimps. These six stages represent the major external landmarks in the regeneration of a claw and were used as markers in the present study.

Simultaneous snapper and pincer autotomy

Although earlier experiments (Darby, 1934; Govind *et al.*, 1986) had shown that simultaneous autotomy of both claws results in the regeneration of the paired claws in their previous configuration, these studies did not report on the growth of the limb buds. We repeated this experiment by autotomizing the paired claws within minutes of each other, one or two days after a shrimp had molted, and monitoring limb bud growth in the ensuing intermolt. Limb bud growth was qualitatively similar on the two sides and followed the criteria listed above. Moreover, the regenerate limb buds of the two sides were also similar in length throughout the intermolt (Fig. 3). At specific times during the intermolt, either the snapper or pincer bud would be slightly more advanced, but there was no consistent pattern and frequently both buds were equal in size. When these limb buds unfolded as claws at the next molt (Fig. 1B), they were much smaller than their predecessors (Fig. 1A) but otherwise similar in morphotype. In all 16 of the 27 animals that successfully regenerated paired claws, the previous asymmetric configuration was retained (Fig. 4A).

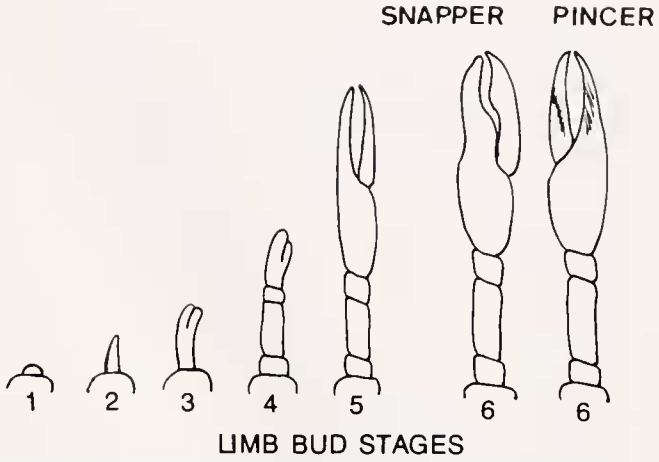

Figure 2. Stages in the regeneration of a limb bud at the site of an autotomized claw in adult snapping shrimps. The development of the limb bud, although a continuous process, has been categorized into six (1–6) separate stages based on external landmarks; at stage 6 the limb buds are differentiated into snapper and pincer types (see text for description of each stage).

Snapper autotomy followed by pincer autotomy

In this experiment, the snapper claw was autotomized one or two days after the shrimp had molted, and the pincer claw was then autotomized at different stages of limb bud regeneration on the snapper side. Thus, the pincer was removed at each stage of limb bud growth classified as stages 1 to 5. Of the 33 animals in which the pincer claw was autotomized at different limb bud stages, 24 animals successfully regenerated both claws. In all cases, the paired claws mimicked their previous configuration.

In a few additional trials, the pincer claw was also removed when the limb bud at the snapper site was at stage

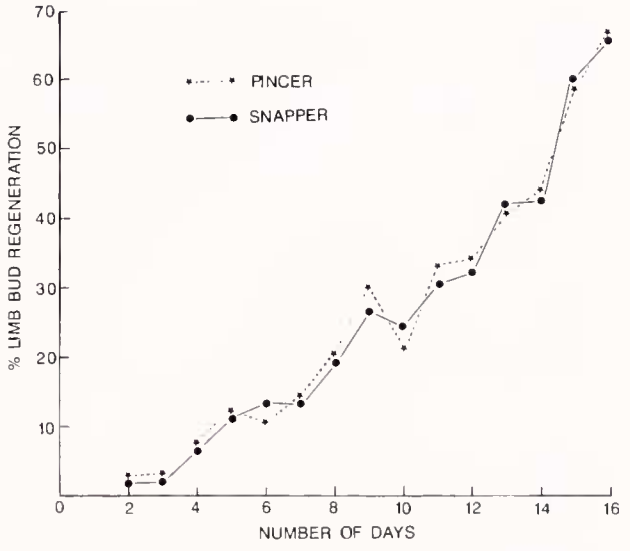

Figure 3. Percent regeneration of snapper and pincer limb buds in adult shrimps following simultaneous autotomy of both claws. Percent regeneration was obtained as follows: (limb bud length/carapace length) × 100. A total of 128 limb buds were measured from 16 animals; each data point represents an average of 4–5 measurements.

6 and already differentiated into a pincer claw. In these animals, a pincer appeared at the old snapper site, but a claw failed to form at the old pincer site because there was not enough time for limb regeneration.

Thus, allowing a limb bud to develop to an advanced stage at the snapper site before the pincer is removed leads to the regeneration of the paired claws in their previous configuration (Fig. 4B). In effect, this is similar to simultaneous autotomy of the paired claws, where the limb buds form at equivalent rates on the two sides and claw asymmetry is retained as described above.

Pincer autotomy followed by snapper autotomy

In this experiment, the snapper claw was autotomized at different stages of limb bud regeneration on the pincer side following autotomy of the pincer claw. At the next molt the morphotype of the newly regenerated paired claws was assessed in terms of retention or reversal of claw asymmetry. The results (Table I) show that with increasing time interval between removal of the paired claws, and hence increasing limb bud development, claw asymmetry was reversed. In other words, as the limb bud stage advances, the proportion of claw reversal increases until 100% reversal is reached at stage 3–5 limb buds.

Removal of the snapper claw when the limb bud is already sufficiently advanced to be discernible as a pincer type (stage 6) does not result in transformation; the limb bud at the pincer site unfolds as a pincer claw, whereas the snapper site lacks a newly regenerated limb or limb bud because of insufficient time for regeneration.

The results of this series of experiments may be summarized as follows (Fig. 4C): removal of the snapper claw when a limb bud of stage 1–2 is present at the pincer site results in retention of claw asymmetry at the next molt in 50% or more of the experimental animals, but removal of the snapper claw when a limb bud of stage 3–5 is present at the pincer site results in reversal of claw asymmetry in all of the experimental animals.

Discussion

When the paired claws were removed sequentially within an intermolt, they regenerated in their previous configuration if the snapper was autotomized first and a limb bud allowed to form at this site before the pincer was autotomized, providing there is enough time to regenerate a claw at the second site. This is the same as when the paired claws are autotomized simultaneously, the limb buds on the two sides regenerate at equivalent rates, and claw asymmetry is retained.

The paired claws also regenerate in their previous configuration when the pincer is removed first and an early limb bud (stage 1–2) is allowed to form before the snapper is removed in 50% or more of the animals. This is equivalent to removing both claws at the same time. However, paired claws regenerate in the reversed configuration in 100% of the animals when the pincer is removed and a more advanced limb bud (stage 3–5) is allowed to form at this site before the snapper is removed. This is equivalent to removing the snapper in the presence of an intact pincer, in which case the existing pincer claw transforms into a snapper at the next molt and a new pincer regenerates at the old snapper site. A stage 3–5 limb bud at the pincer site therefore acts like an intact, fully formed pincer claw in that they both transform into a snapper claw in response to removal of the contralateral snapper claw. In other words, a stage 3–5 limb bud is a suitable target for transformation.

Stage 3 limb buds are characterized by a longitudinal furrow marking the beginning of division of the two most distal segments, the dactyl and propus. The more proximal segments—carpus, merus, and basi-ischium—are delineated in stage 4 buds, and segmentation is complete with final delineation of the dactyl and propus in stage 5 limb buds. Although the most advanced stage 4 and 5 limb buds resemble an intact pincer limb in possessing all of the segments, stage 3 buds with just the beginning of segmentation least resemble an intact limb; yet a stage 3 bud may be signaled into developing as a snapper. It would appear that the transforming signal released with autotomy of the snapper claw can influence the differentiation of tissue, not only in intact pincer claws, but also in a developing limb bud at the pincer site. Thus, for example, the intact pincer claw has a closer muscle whose mixture

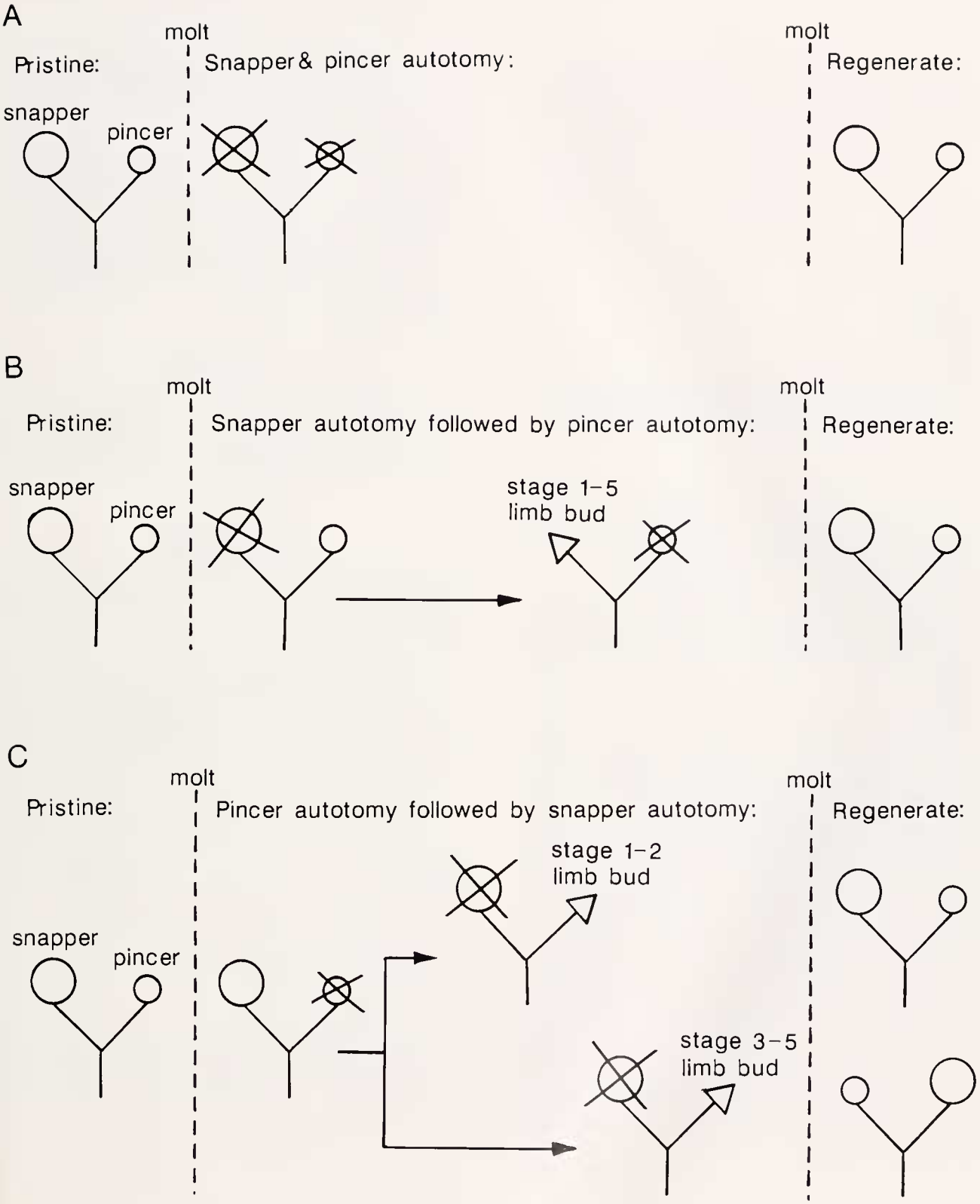

Figure 4. Pictorial representation of the configuration of the paired, asymmetric pincer and snapper claws of adult snapping shrimps in the following experiments. (A) Pincer and snapper claws autotomized simultaneously; regenerate claws appeared in the pristine configuration. (B) The snapper claw was autotomized and, at different limb bud stages at this site, the contralateral pincer claw was autotomized; the regenerate claws appeared in the pristine configuration. (C) The pincer claw was autotomized and, at different stages in the formation of a limb bud at this site, the snapper claw was autotomized; the regenerate claws appeared in the pristine configuration with stage 1–2 buds and in the reversed configuration with stage 3–5 limb buds.

Table I

Configuration of claw asymmetry, whether retained in the previous configuration or reversed, following autotomy of first the pincer claw and then the snapper claw during a single intermolt in adult snapping shrimps

Limb bud stage	Asymmetry retained		Asymmetry reversed	
	Number	%	Number	%
<1.0 (7/10)	6	86	1	14
1.0–1.5 (10/12)	5	50	5	50
2.0–2.5 (7/10)	3	42	4	58
3.0–4.0 (5/8)	0	0	5	100
>4.5 (9/12)	0	0	9	100

The pincer was removed first; then, at various stages of its limb bud development, the snapper was removed. Number in brackets shows the number successful over the number of trials.

of fast and slow fibers is transformed into purely slow fibers of a snapper type by selective death of the fast fibers (Mearow and Govind, 1986) and transformation of the slow fibers from pincer to snapper type (Mellon and Stephens, 1980). In contrast, in a stage 3 limb bud with just the beginning of segmentation, a fully formed closer muscle is unlikely to be present, yet its subsequent development is directed toward snapper muscle rather than pincer muscle.

Shrimps in which the snapper is autotomized after a stage 3–5 limb bud has formed on the pincer site regenerate a new pincer at the snapper site, resulting in reversal of claw asymmetry. The regeneration of a pincer claw at a snapper site requires explanation. Both Wilson (1903) and Darby (1934) considered the possibility that the pincer claw represents a progressive stage in the development of a snapper claw and that inhibition from the contralateral snapper claw can arrest its development. Hence, when the inhibition is removed with snapper autotomy, the pincer continues its development into a snapper, which at the same time restricts claw regeneration to a pincer type on the opposite side. In this way, bilateral asymmetry of the paired claws is ensured. This hypothesis, involving a cross-inhibitory mechanism, would explain why a pincer regenerates at the old snapper site during claw reversal in the present experiments—transformation of the limb bud into a snapper would restrict regeneration to a pincer claw on the opposite side. The hypothesis would also be tenable in cases where only the pincer claw is autotomized, because the intact snapper claw would restrict regeneration to a pincer claw at the autotomy site. However, the hypothesis is insufficient to explain the case in which paired claws are autotomized simultaneously and the regenerate claws appear in their previous configuration. With paired simultaneous autotomy, an additional mechanism would have to be invoked to allow the transforming signal to act at the old snapper site—either by preferentially channeling the signal to this site or by having receptors for the signal exclusively at this site.

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